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Enzymatic generation of whey protein hydrolysates under pH-controlled and non pH-controlled conditions: Impact on physicochemical and bioactive properties

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Abstract

Enzymatic hydrolysis of whey protein (WP) was carried out under pH-controlled and non pH-controlled conditions using papain and a microbial-derived alternative (papain-like activity). The impact of such conditions on physicochemical and bioactive properties was assessed. WP hydrolysates (WPH) generated with the same enzyme displayed similar degree of hydrolysis. However, their reverse-phase liquid chromatograph mass spectrometry peptide profiles differed. A significantly higher oxygen radical absorbance capacity (ORAC) value was obtained for WP hydrolysed with papain at constant pH of 7.0 compared to the associated WPH generated without pH regulation. In contrast, there was no significant effect of pH regulation on dipeptidyl peptidase IV (DPP-IV) properties. WP hydrolysed with the papain-like activity under pH regulation at 7.0 displayed higher ORAC activity and DPP-IV inhibitory properties compared to the associated WPH generated without pH regulation. This study has demonstrated that pH conditions during WPH generation may impact on peptide release and therefore on WPH bioactive properties.

Key words: pH stat; whey proteins; peptide profile; DPP-IV inhibition; ORAC

1 Introduction

Food protein-derived peptides have significant industrial relevance as they are associated with health benefits such as antidiabetic, antioxidant and antihypertensive properties (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo & Recio, 2014; Korhonen & Pihlanto, 2006; Li-Chan, 2015; Nongonierma & FitzGerald, 2015; Power, Jakeman & FitzGerald, 2013). The main approach used to generate bioactive peptides is by enzymatic hydrolysis, however, the outcome in term of the peptides released may be highly dependent on the processing conditions employed (Wei & Zhimin, 2006; Whitehurst & Law, 2002). Indeed, parameters such as the enzyme preparation, protein concentration, pH, duration of the process and incubation temperature have been shown to influence the peptide composition of hydrolysates. Therefore, the processing condition used for the generation of protein hydrolysates can impact the release of peptides and thus may alter the bioactive profile of the resultant hydrolysate (van der Ven, Gruppen, de Bont & Voragen, 2002).

pH is an important parameter which can vary during the hydrolysis process, as the peptide bonds cleavage can modify the buffering capacity of the solution (Wei, et al., 2006). However, the pH can be regulated throughout hydrolysis using a pH stat approach. This strategy allows maintenance of the pH at the enzymes' optimum throughout the reaction. Nevertheless this method involves the addition of a titrant (acid or alkaline) to maintain the target pH. This results in additional salts in the final hydrolysates. That is why an alternative strategy commonly employed in industries consists of adjusting the initial pH to the enzymes' optimum and then allowing the pH to change during the course of the hydrolysis reaction without further addition of a titrant. However, as the tertiary structure of enzymes is pH dependant, even small changes in pH during the course of a non pH-controlled reaction may affect the conformation of the enzyme. As a result, this may affect its hydrolytic activity

(Whitehurst, et al., 2002). For instance, hydrolysis of β-lactoglobulin by trypsin at optimal pH (pH 7.8) and at lower pHs showed few differences in the generation of peptides and also in the kinetics of peptide release (Cheison, Lai, Leeb & Kulozik, 2011). It was also shown that the extent of whey protein isolate hydrolysis with trypsin was dependent on the pH (Cheison, Leeb, Toro-Sierra & Kulozik, 2011). Indeed, altering the pH allowed to modify the proportion of hydrolysed α-lactalbumin, β-lactoglobulin variant A or B after 5 min hydrolysis.

Food-grade industrial enzyme preparations are derived from a wide range of animal, microbial and plant sources. Therefore, these preparations mostly contain mixtures of enzyme activities, each possessing their own unique properties and optimum pH values (Whitehurst, et al., 2002). For this reason, the specific enzymatic activity of a preparation may vary with pH leading to the generation of different peptides. The resultant change in peptide profile may impact on the hydrolysate physicochemical and bioactive properties.

The aim of this study was to compare the impact of whey protein (WP) proteolysis, under pH control and uncontrolled conditions, on the physicochemical and bioactive properties of the food protein hydrolysates. The WP substrate was hydrolysed with two different food-grade enzymatic preparations. Physicochemical properties of the resulting WP hydrolysates (WPH) were then analysed. Their peptide profile and degree of hydrolysis (DH) were studied. With regard to bioactivity, the inhibition of dipeptidyl peptidase IV (DPP-IV) was employed as an *in vitro* indicator of an antidiabetic effect as inhibition of this enzyme is a target for the improvement of serum glucose regulation in type-2 diabetes (Juillerat-Jeanneret, 2014; Tulipano, Faggi, Nardone, Cocchi & Caroli, 2015). Moreover, as a high oxidative status may occur in type-2 diabetic patients, the antioxidant capacity of the hydrolysates generated was analysed (Rani & Mythili, 2014). Consequently, the novelty of this study was to highlight the impact of pH controlled vs. non pH controlled conditions

regarding WPH physicochemistry, antioxidant and antidiabetic bioactivities, as it has not been systematically studied, to our knowledge.

2 Materials and methods

2.1 Reagents

The WP substrate (81% *w/w* protein) was obtained from Carbery Group (Ballineen, Ireland). Gly-Pro-pNA, diprotin A (IPI), porcine DPP-IV (≥ 10 units mg^{-1} protein), trifluoroacetic acid (TFA), tris(hydroxymethyl)aminomethane (TRIS), Trolox, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) radical, mass spectrometry (MS) grade water and acetonitrile (MeCN) were purchased from Sigma Aldrich (Dublin, Ireland). Hydrochloric acid (HCl), sodium hydroxide (NaOH) high pressure liquid chromatography (HPLC) grade water and MeCN were provided by VWR (Dublin, Ireland). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was obtained from Pierce Biotechnology (Medical Supply, Dublin, Ireland).

Two food-grade proteolytic preparations were used in this study as they were previously shown to yield quinoa protein hydrolysates with similar physicochemical and bioactive properties (Nongonierma, Le Maux, Dubrulle, Barre & FitzGerald, 2015). These enzyme preparations consisted of a proteinase preparation from *Carica papaya* latex (papain, activity: 100 papain TU/mg,) and its microbial-derived alternative (papain-like, activity: 154 casein protease units/g), both from Biocatalysts (Cefn Coed, Wales, UK). Each preparation contained several proteinases with broad specificities. Both had similar optimum condition ranges, with pH of 5.0 and 7.5, and temperature of 50 and 70ºC.

All other chemicals were obtained from Sigma Aldrich and were of analytical grade unless otherwise stated.

2.2 Enzymatic hydrolysis of WP

Hydrolyses were carried out as described by Nongonierma, Le Maux, et al. (2015) with minor modifications. Briefly, WP was resuspended at 5% (*w/w*) in distilled water and rehydrated at 50ºC for 30 min under agitation. The solutions were adjusted to the desired pH (see below) using NaOH (0.5 M) or HCl (0.1 M). Two different enzyme preparations were used to hydrolyse WP: a papain preparation (P) and its microbial-derived alternative (papain-like, PL). The enzyme was added at an enzyme to substrate (E:S) ratio of 2% (*v/w*) and hydrolysis was carried out at 50ºC for 3 h under agitation. Hydrolysis was terminated by heat inactivating the enzyme in a water bath at 90ºC for 20 min or 100ºC for 40 min for PL and P, respectively. The hydrolysates generated with the papain (WPH-P) and with the papain-like enzyme (WPH-PL) were then freeze-dried (FreeZone 18L, Labconco, Kansas City, MO, USA) and stored at -20ºC prior to further analysis.

Hydrolyses reactions carried out under pH stat (controlled pH) conditions (Titrando 843, Tiamo 1.4 Metrohm, Dublin, Ireland) were initially adjusted and maintained at pH 7.0 for both enzyme preparations. The hydrolysis reactions carried out without pH control were also initially adjusted to pH 7.0. On termination of the reaction, it was observed that the pH had dropped to 6.1 and 6.3 for the WPH-PL and WPH-P, respectively. Therefore, hydrolysis was also carried out under pH controlled conditions at pH 6.1 for PL and 6.3 for P. Each hydrolysis reaction was performed in triplicate.

2.3 Quantification of the degree of hydrolysis (DH) using the trinitrobenzenesulfonic acid (TNBS) method

The degree of hydrolysis (DH) was quantified using the TNBS method according to Adler-Nissen (1979) and as described by Hernández-Herrero, Roig-Sagués, López-Sabater, Rodríguez-Jerez and Mora-Ventura (1999) with modifications. Samples (0.25-1.00 g L^{-1})

were prepared in 1% (*w/v*) sodium dodecyl sulphate (SDS) solution and incubated at 50ºC for 1 h. Samples (10 µL) were added to 80 µL of sodium phosphate buffer (0.2125 M, pH8.2) and 80 µL of 0.1% (*w/v*) TNBS in a 96-well microplate. The microplate was incubated at 50ºC and the absorbance was read at 420 nm every 5 min for 1 h using a plate reader (Biotek Synergy HT, Winoosky, VT, USA). Leucine was used as a standard (at concentrations ranging from 0 to 4 mM) to allow determination of the free amino group content of the samples (AN, in mg N mg^{-1} sample). DH was measured using the following formula:

$$\%\, DH = \frac{AN_{WPH} - AN_{unhydrolysed\ WP}}{Npb} \times 100$$

A value of 123.3 mg g^{-1} was used as the nitrogen content of the peptide bonds (Npb) for WP (Spellman, McEvoy, O'Cuinn & FitzGerald, 2003). Each sample was analysed in triplicate.

2.4 Sample analysis by polyacrylamide gel electrophoresis

WP and WPH were analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Mini-PROTEAN TGX precast Gels (4-20 % resolving gel, Bio-Rad Laboratories Inc., Hercules, CA) were used on a Mini Protean II system (Bio-Rad) according to the manufacturer's instructions. Samples were prepared under reducing conditions (with β-mercaptoethanol). A wide range molecular weight calibration kit (6,500-200,000 Da, Sigma Aldrich) was used as molecular weight standards.

2.5 Analytical chromatography

Gel permeation high performance liquid chromatography (GP-HPLC) was used to determine the molecular mass distribution at 214 nm of the hydrolysates as described by Nongonierma and FitzGerald (2012).

2.6 Sample analysis by reverse phase ultra-performance liquid chromatograph mass spectrometry (RP-UPLC-MS/MS)

Samples were analysed on RP-UPLC-MS/MS, using a Waters Acquity UPLC system (Waters, Milford, MA) coupled to an Impact HD (Quadrupole, Time-of-Flight) mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) as described by Le Maux, Nongonierma, Murray, Kelly and FitzGerald (2015). Briefly, the UPLC system was equipped with a tunable UV detector set at 214 and 280 nm. The mass spectrometer was fitted with an electrospray ionisation (ESI) source and was used in the positive ion mode. Instrument control and data acquisition were performed using Hystar software (Bruker Daltonics GmbH) as described by Le Maux, et al. (2015)

Data were analysed using ProfileAnalysis (Bruker Daltonics GmbH). Visualisation of peptide peak intensities was carried out with Venn diagrams using the venn function (Version 1.7) on Matlab R2015a (The MathWorks Inc., Natick, MA). Only peaks with a high intensity (threshold of 3×10^5) were analysed. The number of peptides with similar intensities for all samples represented in the diagram were reported at the intersection of all the samples. Peptide peaks having different intensities (4 fold differences and *P*-value of 0.05) were reported in the zone representing the hydrolysates which had the highest intensity for these peaks.

2.7 DPP-IV inhibition assay

The DPP-IV inhibitory activity of each sample was determined as described by Nongonierma and FitzGerald (2013a). The WPH were resuspended in HPLC water at a concentration ranging from 2.5 10^{-3} to 2.5 mg mL^{-1}. The DPP-IV IC_{50} values were determined by plotting the percentage inhibition as a function of the WPH concentration expressed in mg dry powder mL^{-1}. Each sample was analysed in triplicate.

193

194 *2.8 Determination of the antioxidant capacity using the oxygen radical absorbance*
195 *capacity (ORAC) assay*

196 The antioxidant capacity of each sample was determined using the ORAC assay as
197 per Zulueta, Esteve and Frígola (2009), with some modifications described by Nongonierma,
198 Le Maux, et al. (2015). WP and WPH were tested at a final concentration of 8 mg L^{-1}. The
199 ORAC values were expressed as μmol of Trolox equivalents (TE) per g of dry powder. Each
200 sample was analysed in triplicate.

201

202 *2.9 Statistical analysis*

203 Results are presented as the mean of triplicate determinations ± SD. Results were
204 compared with R software 3.1.0 package (R Foundation for Statistical Computing, Vienna,
205 Austria) using the Rcmdr library version 2.1-7 and an ANOVA system with a Tukey's test at
206 a significance level $P < 0.05$.

207

208 # 3 Results

209

210 *3.1 Degree of hydrolysis of WPH*

211 The DH of the WPH samples are presented in Table 1. All the WPH-P were of the
212 same order of % DH ($P > 0.05$). Likewise, there was no significant difference between all the
213 WPH-PL samples ($P > 0.05$). However, the WPH-PL were significantly more hydrolysed
214 than the WPH-P ($P < 0.05$), with DH of 4.93 ± 0.06 % and 10.80 ± 0.32 % for WPH-P-pH 7
215 and WPH-PL-pH 7, respectively.

216

3.2 Molecular mass distribution of WP samples by GP-HPLC and polyacrylamide gel electrophoresis

Protein hydrolysis was observed by GP-HPLC, which highlighted the breakdown of the high molecular mass (> 10 kDa) components within all the WPH samples as compared to WP (Figure 1). The GP-HPLC peptide profiles showed a higher proportion of short peptides (< 1 kDa) in the WPH-PL than in the WPH-P samples. WPH-P-pH 7 had the highest proportion of high molecular mass (26.8 % of compounds > 10 kDa) and WPH-PL-pH 7, the lowest (11.1 % of compounds > 10 kDa).

Similar results were observed by reducing SDS-PAGE analysis, showing greater protein hydrolysis in WPH-PL compared to WPH-P samples. Moreover, bands of intact proteins, such as bovine serum albumin (BSA) at 66 kDa, could only be observed in the WPH-P samples (Supplementary data Fig. S1).

3.3 Peptide profile of WPH by RP-UPLC-MS/MS

All replicates of the same WPH had similar peptide profiles (data not shown). Furthermore, WPH samples produced with the same enzyme preparation had comparable peptide profiles, with certain peaks displaying different intensities under certain pH conditions (some examples are indicated by red dashed boxes in Figure 2). These differences were presented using Venn diagrams for each enzyme. The number of peptides with high peak intensity (threshold of 3×10^5) was 238 and 236 for the WPH-P and WPH-PL samples, respectively. The Venn diagrams showed that most peptides with high intensity were common in hydrolysates generated at the three pH conditions (169 and 176 common peptides for WPH-P and WPH-PL samples, respectively, Figure 3). These peptides generally had the same intensity in the three WPH samples produced with the same enzyme. Sixty-nine peptides, ranging from 519.308 to 1315.228 *m/z* showed intensity differences between the

242 WPH-P samples. For example, the highest intensity peak (657.869 *m/z* and elution time of
243 62.7 min) had intensities of $10.0 \pm 0.5 \times 10^6$, $5.2 \pm 0.8 \times 10^6$ and $2.0 \pm 0.5 \times 10^6$ for WPH-P-
244 no pH stat, WPH-P-pH 6.3 and WPH-P-pH 7, respectively. Therefore, this peptide was
245 reported in the Venn diagram for the WPH-P-no pH stat sample (Figure 3A), among the
246 twenty-one peptides that had their highest intensities for WPH-P-no pH stat. However, no
247 clear pattern could be observed concerning the effect of each pH condition on the intensity of
248 the peptides released. Similar deductions could be proposed for the WPH-PL samples, which
249 had 60 peptides with different intensities ranging from 519.374 to 1451.066 *m/z*.
250
251 *3.4 Bioactive properties*
252 The DPP-IV IC_{50} values were significantly lower for all the WPH compared to
253 unhydrolysed WP ($P < 0.05$, Table 1). All the WPH-P samples were of the same IC_{50} order
254 ($P > 0.05$) with the lowest IC_{50} at 1.40 ± 0.12 mg mL^{-1} for WPH-P-pH 6.3. The IC_{50} of the
255 pH stat samples, WPH-PL-pH 7 and WPH-PL-pH 6.1, were significantly lower than the
256 value for their non pH stated equivalent (WPH-PL-no pH stat) ($P < 0.05$), with IC_{50} of $1.01 \pm$
257 0.09 and 1.82 ± 0.26 mg mL^{-1} for WPH-PL-pH 6.1 and WPH-PL-no pH stat, respectively.
258 The pH stated WPH-PL-pH 7 sample had the lowest IC_{50} values of all the WPH (IC_{50} of 0.72
259 $\pm$ 0.08 mg mL^{-1}, $P < 0.05$).
260 The ORAC values of all the WPH were significantly higher than that of unhydrolysed
261 WP ($P < 0.05$, Table 1). There was no significant difference between the ORAC values of the
262 WPH-PL samples ($P > 0.05$). The ORAC value of the WPH-P-no pH stat sample was
263 significantly lower than that of WPH-P-pH 7 ($P < 0.05$), with values of 192.54 ± 42.61 and
264 285.32 ± 36.71 µmol TE g^{-1} protein equivalent, respectively.
265

266 # 4 Discussion

The use of pH regulation to generate protein hydrolysates is important in laboratory environments to control enzymatic hydrolysis reactions. However, in an industrial context, pH regulation during hydrolysis may need to be avoided in order to (i) minimise the inclusion of additives, (ii) avoid possible contaminations and (iii) simplify the hydrolysis protocols. A previous study reported a 22 % decrease in *in vitro* insulin secretion by pancreatic β-cells following stimulation with a WPH generated without pH regulation as compared to a WPH generated with pH regulation (Nongonierma, Gaudel, et al., 2013). However, to our knowledge, the impact of WPH generation in pH controlled vs. non pH controlled conditions has not been systematically studied in the context of antioxidant and DPP-IV inhibitory bioactivities. Therefore, this study investigated the generation of WPH in different pH environments and analysed their impact on hydrolysates bioactivities and physicochemical properties.

The ORAC values obtained were shown to be dependent on pH conditions used to generate the hydrolysates. The higher ORAC values were observed for hydrolysates generated at the constant optimal enzyme pH (i.e., pH 7.0) for papain and papain-like enzyme preparations. The hydrolysis generated under pH stat conditions also displayed more potent the DPP-IV inhibitory activities compared to non-pH stat hydrolysates. The bioactive differences between samples generated under different pH conditions may be due to pH-dependant changes in enzymes conformation (Whitehurst, et al., 2002). Indeed, alteration of enzymatic activity under different pH conditions may have influenced the peptides released. Therefore, this study has highlighted the importance for considering the use or otherwise of pH controlled conditions depending on the applications of the hydrolysates, i.e., generation of the more potent bioactive hydrolysate or importance of reducing the additives incorporation.

Many studies have reported ORAC values and *in vitro* DPP-IV inhibitory activity for milk protein hydrolysates (Di Pierro, O'Keeffe, Poyarkov, Lomolino & FitzGerald, 2014; Lacroix & Li-Chan, 2014; Nongonierma & FitzGerald, 2013a). The ORAC values reported herein were of the same order as in previous studies. For instance, Power (2013) reported similar ORAC values for WPH hydrolysates. The DPP-IV IC_{50} obtained in this study were also of the same order as those in the literature. For example, IC_{50} of 1.43 ± 0.27 mg mL^{-1} and 1.33 ± 0.17 mg mL^{-1} were previously reported for WPH generated with food-grade gastrointestinal enzyme preparations and Corolase PP, respectively (Nongonierma & FitzGerald, 2013a, 2013b)

Most of peptides observed by LC-MS were common within all WPH samples hydrolysed with the same enzyme preparation. However, some peptides showed different peak intensity in MS depending of the pH conditions employed. These peptides, along with peptides poorly represented in MS may be responsible for both the DPP-IV inhibitory and the ORAC activity variations between samples. The identity of these peptides was not investigated herein, however they all had *m/z* values inferior to 1500 Da. Short milk-derived peptides (< 11 amino acids), containing one or more residues of histidine, proline, tyrosine and tryptophan, have already been reported as displaying antioxidative properties (Pihlanto, 2006). Similarly, most DPP-IV inhibitory peptides reported in the literature to date appear to possess < 8 amino acid residues. In general peptides comprising a Trp at the N-terminus and/or a Pro at position 2 were generally potent DPP-IV inhibitors (Nongonierma & FitzGerald, 2014).

The papain or microbially-derived papain-like enzyme preparations did not display similar WP hydrolysis in the conditions used herein. Indeed, the two preparations yielded

hydrolysates with significant differences such as their physicochemical properties. For example, a higher %DH and different peptide profiles were observed for the WPH-PL compared to the WPH-P samples. The DPP-IV inhibitory activities were also different; however the ORAC activities were of the same order for the samples produced with the papain or papain-like preparations for all pH conditions. Conversely, a previous study showed that these two enzyme preparations allowed the generations of quinoa hydrolysates with similar physicochemical and bioactive properties (Nongonierma, Le Maux, et al., 2015). Thus, the ability of the microbially-derived enzyme, used herein, to have the same properties as papain would seem to be substrate dependant. Indeed, these enzyme preparations were reported by the suppliers to contain several proteinases with broad specificities. Therefore, these enzymes may induce variable peptide cleavages on different protein substrates.

5 Conclusions

WPH generated with or without pH regulation showed similar physicochemical properties. However, some peptides exhibited different peak intensities in LC-MS, depending on the pH condition used during WPH generation. These differences in peptide composition may have contributed to the different bioactive properties of the samples. For example, WP hydrolysed with PL under pH regulation at 7.0 displayed more potent ORAC activity and DPP-IV inhibitory properties compared to the non pH controlled WPH. Consequently, the use of pH regulation vs. no pH regulation during enzymatic hydrolysis of WPs should be carefully considered as it was shown to affect peptide release and consequently the bioactive properties.

Additionally, the difference between pH stat vs. non pH stat hydrolysis conditions should be established for different enzymes and different bioactivities. Further studies are

also required to elucidate the exact role of pH alteration on enzyme kinetics, potential effects on substrate conformation and hydrolysate specificity. Indeed, hydrolysis reaction are conventionally terminated on a time basis. However, as the objective of protein hydrolysis is to generate bioactive peptides, it would be interesting to use the hydrolysate bioactivity as the main parameter to stop the reaction. This could be achieved by having a better understanding of the kinetics of peptide release, which is dependant on conditions such as pH or temperature (Cheison, Leeb, et al., 2011). Finally, assessment of the peptides stability to gastrointestinal digestion is needed to verify that their bioactive properties can translate *in vivo*.

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Conflits of interests

The authors declare that they have no conflict of interest.

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448

Table and Figure captions

Table 1. Percentage of degree of hydrolysis (%DH), half maximum inhibitory concentration (IC_{50}) for DPP-IV and ORAC of WP hydrolysed with papain (WPH-P) and the papain-like enzyme (WPH-PL) under different pH conditions (no pH stat, pH stat at 7, 6.3 or 6.1). The DPP-IV IC_{50} value of IPI, the positive control, was of 3.86 ± 0.50 µM. All values in the table are expressed in dry powder. Values represent the mean ± SD of three replicates (n = 3). For each assay, values with different superscript letters are significantly different ($P < 0.05$). TE: Trolox equivalent, NA: not applicable.

Figure 1. Molecular mass distribution determined by GP-HPLC of WP hydrolysed with papain (WPH-P) and the papain-like enzyme (WPH-PL) under different pH conditions (no pH stat, pH stat at 7, 6.3 or 6.1). Bovine serum albumin, β-lactoglobulin, α-lactalbumin, aprotinin, bacitracin, Leu-trp-Met-Arg, Asp-Glu and tyrosine were used as standards. Values represent the mean ± SD of three replicates (n = 3).

Figure 2. Representative RP-UPLC-MS base peak chromatograms (BPC) of WP hydrolysed with (A) papain (WPH-P) and (B) a papain-like enzyme (WPH-PL) under different pH conditions (no pH stat, black curve; pH stat at 7, dark grey curve; pH stat at 6.3 or 6.1 for WPH-P and WPH-PL respectively, light grey curve). Red dashed boxes indicate some peaks that presented different intensities for the different WPH samples.

Figure 3. Venn diagram visualisation of peptide peaks common and specific to WP hydrolysed with (A) papain (WPH-P) and (B) a papain-like enzyme (WPH-PL) under different pH conditions (no pH stat, blue; pH stat at 7, yellow; pH stat at 6.3 or 6.1 for WPH-

P and WPH-PL respectively, magenta). Peptide peak intensity were assessed by RP-UPLC-MS. Peptides with common intensity for several samples were represented at the intersection of these specific samples.

Supplementary figure 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the whey protein (WP) hydrolysed with papain (WPH-P) and the papain-like enzyme (WPH-PL) in different pH conditions (no pH stat, pH stat at 7, 6.3 or 6.1). Each hydrolysate was generated in triplicates. Samples were displayed as follow: (A) WP, WPH-P-no pH stat, WPH-P-pH 7; (B) WP, WPH-P-pH 6.3, WPH-PL-pH 6.1; (C) WP, WPH-PL-no pH stat WPH-PL-pH 7. Mw, molecular weight markers (6.5, 14.2, 20, 24, 29, 36, 45, 55, 66, 97, 116, 200 kDa).

Table 1

Sample	DH (%)	DPP-IV IC_{50} (mg mL^{-1})	ORAC values (μmol TE g^{-1})
WP	NA	> 2.5	71.52 ± 19.29 [a]
WPH-P-no pHstat	4.31 ± 0.38 [a]	1.54 ± 0.15 [c]	192.54 ± 42.61 [b]
WPH-P-pH7	4.93 ± 0.06 [a]	1.48 ± 0.18 [c]	285.32 ± 36.71 [cd]
WPH-P-pH6.3	5.36 ± 0.50 [a]	1.40 ± 0.12 [c]	231.90 ± 25.40 [bc]
WPH-PL-no pHstat	9.41 ± 0.12 [b]	1.82 ± 0.26 [d]	258.02 ± 21.92 [cd]
WPH-PL-pH7	10.80 ± 0.32 [b]	0.72 ± 0.08 [a]	308.24 ± 56.13 [d]
WPH-PL-pH6.1	8.98 ± 1.49 [b]	1.01 ± 0.09 [b]	249.92 ± 26.41 [bd]

Figure 1

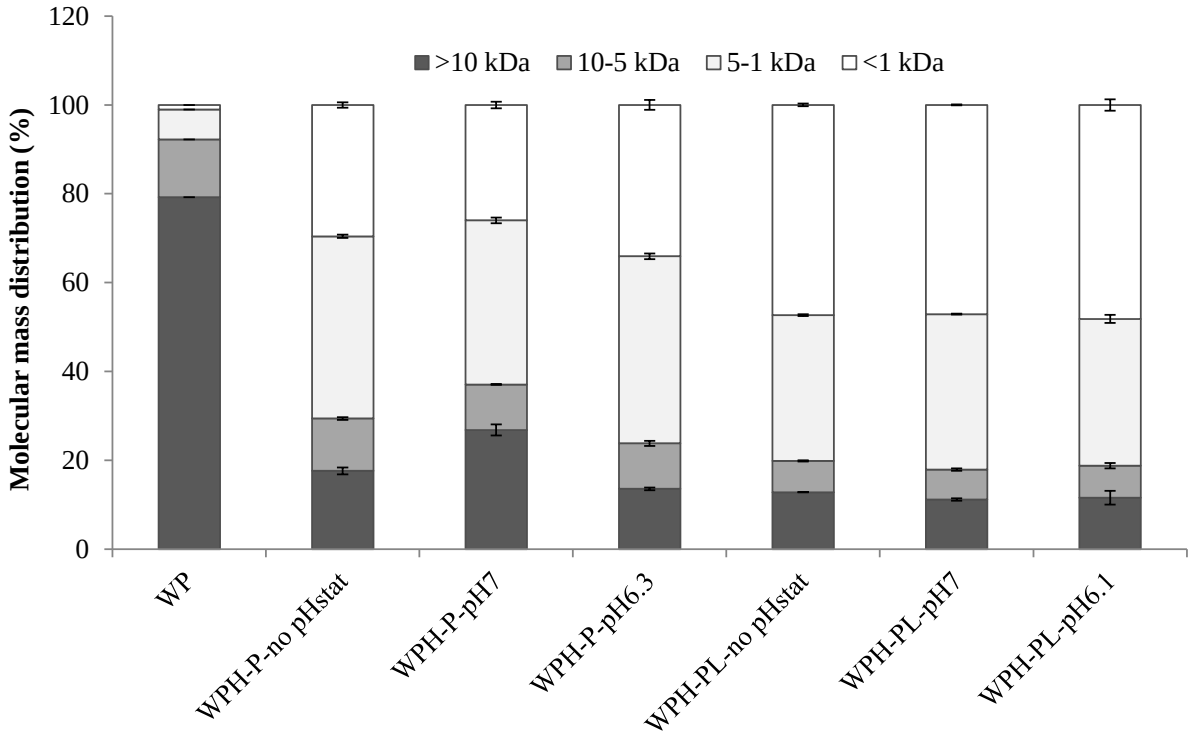
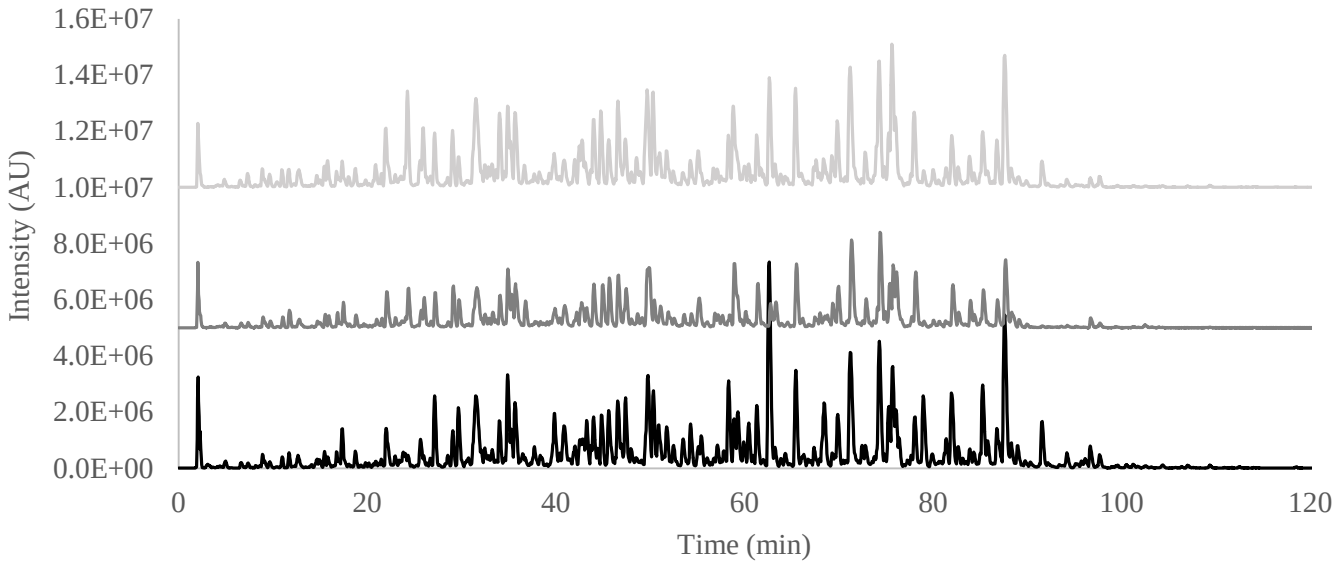

120
100
80
60
40
20
0
Molecular mass distribution (%)
>10 kDa
10-5 kDa
5-1 kDa
<1 kDa
WP
WPH-P-no pHstat
WPH-P-pH7
WPH-P-pH6.3
WPH-PL-no pHstat
WPH-PL-pH7
WPH-PL-pH6.1

Figure 2

A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1.4E+07
1.2E+07
1.0E+07
8.0E+06
6.0E+06
4.0E+06
2.0E+06
0.0E+00
Intensity (AU)
0
20
40
60
80
100
120
Time (min)

B

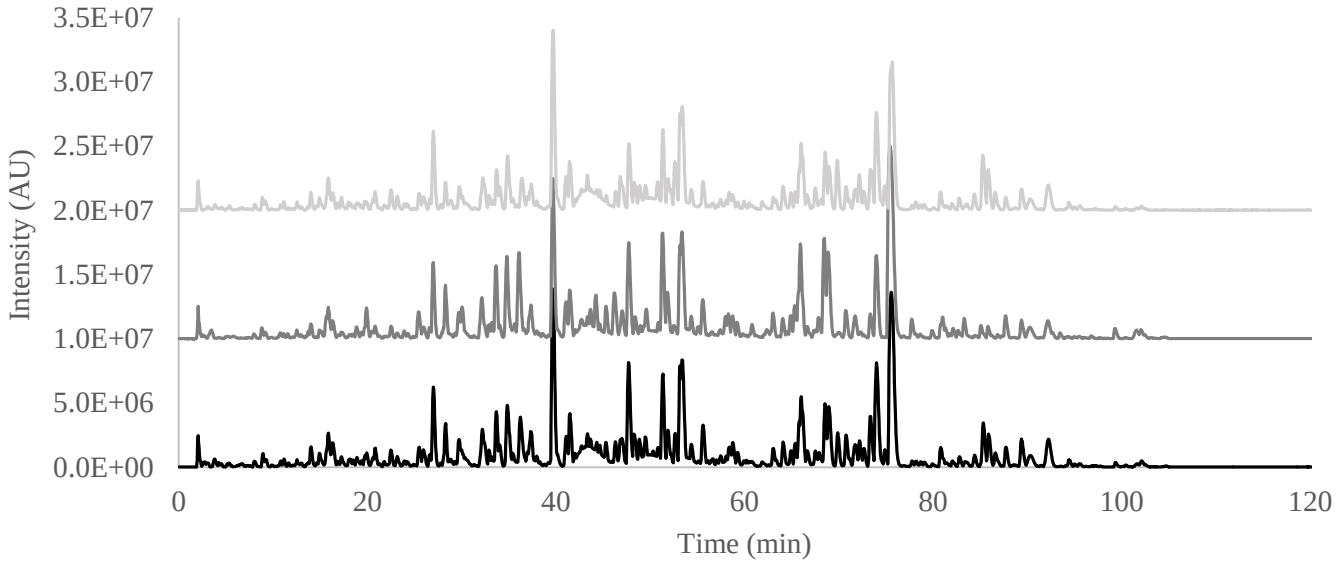
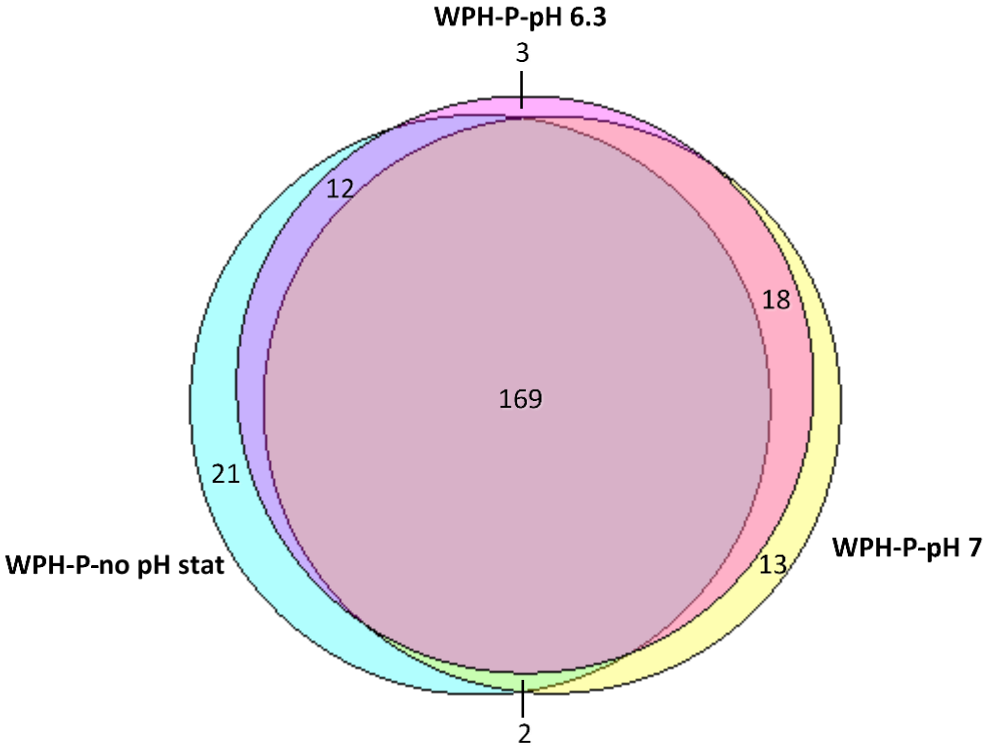
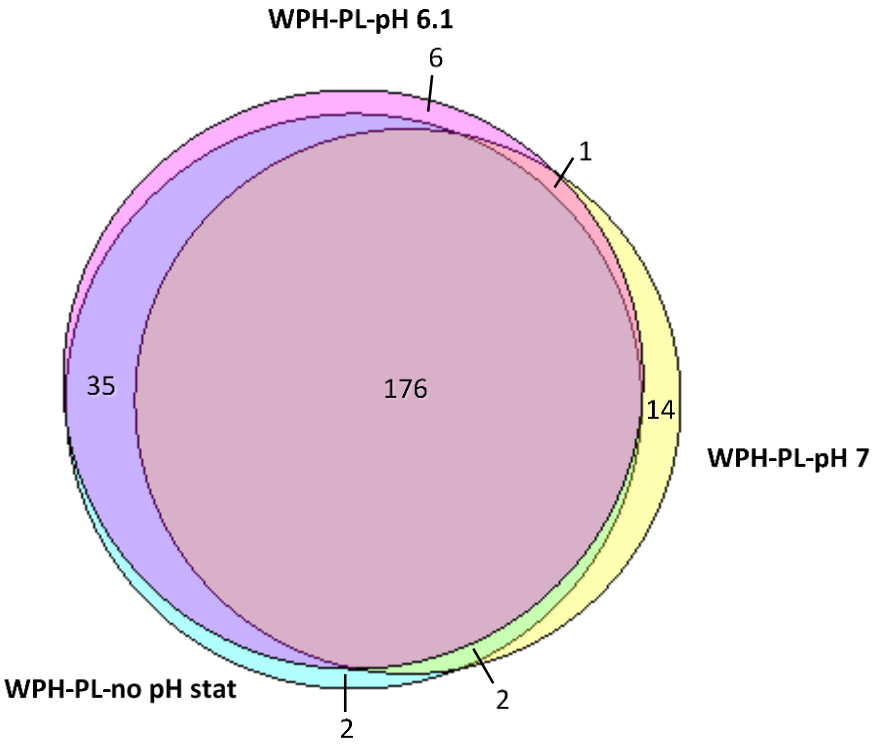

3.5E+07
3.0E+07
2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.0E+06
0.0E+00
Intensity (AU)
0
20
40
60
80
100
120
Time (min)

Figure 3

A

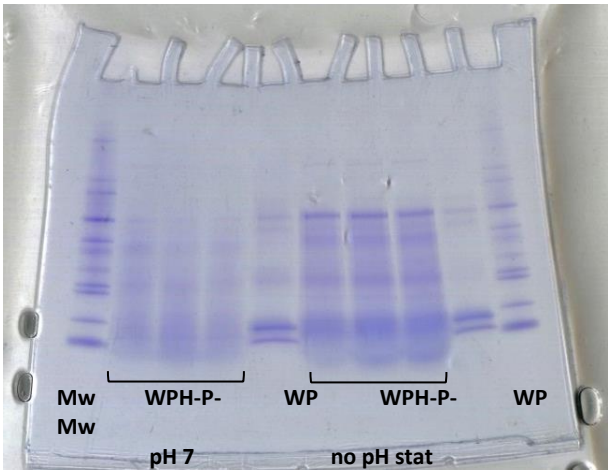

WPH-P-pH 6.3
3
12
18
169
21
WPH-P-no pH stat
13
WPH-P-pH 7
2

B


WPH-PL-pH 6.1
6
1
35
176
14
WPH-PL-pH 7
WPH-PL-no pH stat
2
2

Supplementary figure 1

(A)



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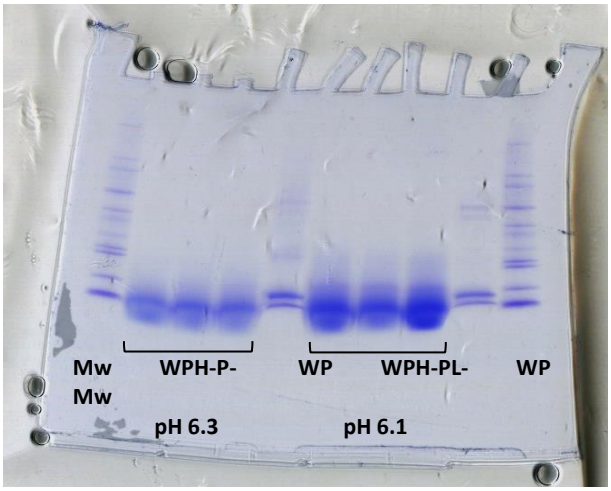

(C)